STUDIES IN THE FORMATION OF ETHYL METHYLPHOSPHONOFLUORIDATE FROM RAT AND HUMAN SERUM EXPOSED TO VX AND IN THE PRESENCE OF FLUORIDE ION

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ABSTRACT

A method has been developed for the analysis of a VX nerve agent biomarker in blood that is very sensitive and selective. VX was detected in spiked human and rat sera by the generation of its corresponding G-series derivative, ethyl methyl phosphonofluoridate (VX-G). This method utilizes a C18 solid-phase extraction (SPE) followed by quantification using a gas chromatograph with either a flame photometric detector (GC-FPD) or a mass spectrometer (GC-MS). VX-G was completely resolved from sarin (GB) and the method has the potential to resolve other nerve agents in the VX series of cholinesterase inhibitors. The method detection limit was 10.5 pg of agent on column.

INTRODUCTION

The ability to verify nerve agent exposure and to determine the degree of exposure is extremely important for medical, tactical, and political reasons. Current verification methods depend on establishing either a significant depression in cholinesterase activity and/or the presence of nerve agent metabolites. Cholinesterase activity is known to fluctuate significantly within a population and even within individuals. A simple correlation between cholinesterase depression and severity of exposure is not always evident. Nerve agent metabolites such as the alkylmethylphosphonates are not easily observable and currently require derivatization for low-level GC analysis which adds time and complexity to the sample analysis scheme. Our goal was to find or develop analytical methods capable of quantifying VX exposure in biological matrices such as blood and tissue using available instrumentation. A literature search for nerve agent methods produced one very promising candidate method which served as a starting point. In this method, GB was regenerated from bound sites in the blood by relatively simple sample matrix manipulation (addition of fluoride at pH 4) followed by solid-phase cartridge extraction.[1] This method has never been used with VX analogs and it may prove useful as a tool to study VX exposures. VX itself would not reform from under the conditions of the assay. The product would likely be methyl ethylphosphonofluoridate.

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EXPERIMENTAL

CHEMICALS AND MATERIALS

Rat and human sera were purchased from Sigma (St. Louis, Missouri). Potassium fluoride (CAS No. 7789-23-3) and sodium sulfate (anhydrous, CAS No. 7757-82-6) from Aldrich Chemical Company (Milwaukee, Wisconsin). Silver fluoride pads were purchased from CMS (OI Analytical, Birmingham, AL). Waters (Waters Associates, Millipore Corp., Milford, MA) Sep-Pak® 500 mg and 200 mg C_{18} solid-phase extraction (SPE) cartridges were used for extractions. Ethyl acetate and 2-propanol were pesticide grade from Aldrich Chemical Company (Milwaukee, Wisconsin). Aqueous acetate buffer (pH 3.5) was prepared from acetic acid (0.189 M) and sodium acetate (10.8 mM). The internal standard was decadeuterated diethyl ethylphosphonate which was synthesized at ECBC using standard methods.

SAMPLE PREPARATION

Human and rat sera (Sigma, St.Louis, MO) was spiked with dilute VX (in 2-propanol) at 147.3 ng/mL and 176.6 ng/mL, respectively. The exposed serum was filtered using a 500 mg C_{18} SPE cartridge (conditioned first with 1.5 mL 2-propanol and then with 1.5 mL acetate buffer) to separate the free from the protein bound nerve agent. The free agent was eluted with 1.5 mL ethyl acetate, collected over sodium sulfate and saved for analysis. For the standard assay, the resulting spiked human serum was then analyzed in 0.25-mL aliquots by addition of both 0.75-mL acetate buffer and 0.2 mL of potassium fluoride solution. Rat serum required only acetate buffer. The resulting treated serum was extracted using a 200-mg C_{18} SPE cartridge (conditioned first with 1.0 mL 2-propanol and then with 1.0 mL acetate buffer) and the analytes were eluted with 1.0 mL ethyl acetate. Sodium sulfate was added to dry the extract that was then analyzed by GC-FPD. Evaluation of the relative importance of the reagents was assessed by substituting saline for one or both reagents. In addition, extracts in some cases were injected through the silver fluoride pad.

INSTRUMENTAL

Samples were analyzed on either a Hewlett-Packard 5890 GC-FPD or a Hewlett-Packard 6890 GC-5973 MSD (Newark, DE). Sample inlet was by Tenax® solid sorbent tube (Depot Area Agent Monitoring System (DAAMS) tube: Dynatherm Inc, Oxford, PA) using an ACEM 900 (Dynatherm Inc, Oxford, PA) desorber interfaced to the GC column via butt-connector. The GC column was a 30 m x 25 mm x 0.5 um thickness DB-5 MS (J&W Scientific, Avondale, CA). The ACEM 900 temperature program was as follows: Dry 60 ^{o}C for 1 minute, Tube Heat 200^{o}C for 3minutes, Cool for 1minute, Trap Heat 275 ^{o}C for 3 minutes. The GC oven temperature program was as follows: Initial 40 ^{o}C for 2 minutes, ramp to 160 ^{o}C at 15 ^{o}C/minute, ramp to 260 ^{o}C at 40^{o}C/minute and held for 3 minutes. The MSD was used in the electron ionization mode with selected ion monitoring at m/z 111, 99, and 82. After the sample was desorbed on the GC column the sorbent tube was reconditioned by backflushing using 100 mL/min flow of dry nitrogen at 280-300 ^{o}C for five to eight minutes to decrease the high boiling point interference from the serum samples. Backflushing of the sorbent tube prevents degradation of the instrument and column producing a stable baseline despite the complex nature of the sample matrix.

A standard curve was established by sequentially injecting five VX standards from 17.66 ng/mL to 176.6 ng/mL on a silver fluoride pad. The silver fluoride pad was connected to a Tenax® DAAMS tube while a flow of air at approximately 100 mL/min as determined by an inline ball flowmeter was pulled through the system by vacuum. A collection time of two minutes was used to transfer the G-analog from the silver fluoride pad to the DAAMS tube. Prior to analysis, tubes were spiked with 94 pg of internal standard separately using a flow of nitrogen of approximately 100 mL/min. The peak-area-ratio (PAR) for

each standard was plotted against the mass of VX-G injected to yield a linear relationship with a correlation coefficient of greater than 0.99.

RESULTS AND DISCUSSION

Standard curves were created by sequentially injecting five VX standards from 17.66 to 176.6 pg through a silver fluoride impregnated fiber pad. The efficiency of VX conversion to VX-G using this method has been reported to be better than 75% depending on the age and condition of the silver fluoride pad.[2] Noting changes in the daily VX standard results can assess degradation of the pad. Analysis of an 88.3 pg VX standard through a single pad over six days produced a mean recovery of 86.3 pg with a standard deviation of 8.10 and a percent relative standard deviation of 8.29 percent. An estimate of the MDL was obtained by the analysis of seven replicate 20 pg VX injections. The MDL was approximately 10.5 pg of VX.

For this method to be useful, it should be able to distinguish between closely related molecules within the G-series of nerve agents. GB differs by only a methylene group from VX-G and presents an important potential interference. Using this method GB was clearly resolved from the VX-G and would not interfere with the method.

The importance of fluoride ion and acidification were assessed as well as the use of the silver fluoride pad on spiked serum samples. Table 1 lists the analysis results of spiked serum prepared under various conditions of buffer and fluoride ion. Variations of sample preparation and analysis included the following treatments: 1) analysis of the initial 500 mg C_{18} SPE cartridge filter eluent by injecting through silver fluoride pad, 2) substitution of saline for all reagents added to the filtered spiked serum followed by another SPE step, 3) substitution of saline for all reagents added to the filtered spiked serum followed by another SPE step and injection through a silver fluoride pad, 4) addition of buffer and saline (no KF) to the filtered serum followed by another SPE step, 5) addition of buffer and saline (no KF) to the filtered serum followed by another SPE step and injecting through a silver fluoride pad, 6) addition of KF and saline (no buffer) to the filtered serum followed by another SPE step, 7) addition of KF and saline (no buffer) to the filtered serum followed by another SPE step and then injecting through a silver fluoride pad, and 8) addition of KF and buffer to the filtered serum followed by another SPE step.
According to Table 1, treatments six and seven indicated that the addition of buffer to adjust the pH to approximately four was not absolutely necessary to produce some VX-G if fluoride was present in large abundance. Only approximately 47% and 85% as much VX-G was formed without pH adjustment in the rat serum and human serum, respectively (rat: [1.85/3.95]100=46.8%, human: [3.06/3.60]100=85%) for treatment six. The effect of fluoride ion on the spiked human serum was noticeably greater than on the rat serum possibly because rat serum has significant levels of endogenous fluoride.

TABLE 1. VX-G Recovered (ng/mL) from Rat and Human Sera Spiked with VX.

	ng/mL VX-G Recovered							
Treatment	1 Excess VX on Filter	2 Saline only	3 Saline + Pad	4 Saline + Buffer	5 Saline + Buffer + Pad	6 Saline + KF	7 Saline + KF+ Pad	8 Buffer + KF
Rat Serum	165*	0	0	3.29	***	1.85	1.68	3.95
Human Serum	88.2**	0	5.63	0	4.05	3.06	12.7	3.60

* Spike level = 176.6 ng/mL, injected through silver fluoride pad.
** Spike level = 147.3 ng/mL, injected through silver fluoride pad.
*** Rat serum not analyzed

The results when the extract of the human serum was not treated with KF but injected through the AgF pad indicated a substantial amount of VX-G was formed without the presence of aqueous fluoride ion. Since the source of fluoride in these cases was provided by the silver fluoride pad, a precursor of VX-G must have been present in the ethyl acetate extract. The precursor could have been VX or a breakdown product of VX that was still capable of reacting on the silver fluoride pad to generate VX-G. Excess VX was extracted using a 500 mg C18 SPE cartridge the same as was used with the rat serum. Moreover, the amount spiked in the case of the human serum was less than that of the rat serum therefore the capacity of the SPE cartridge was likely not exceeded. Proportionately less excess VX was recovered from the initial SPE cartridge with human serum than the rat serum which indicated that the VX was either binding to sites in the human serum and being carried through the SPE cartridge or that VX was degrading faster in human serum. However, the degradation product was still capable of forming VX-G when it came in contact with the silver fluoride pad and/or aqueous fluoride ions. In theory, any breakdown product of VX that retains the general O-ethyl methyl phosphonothioate structure can produce VX-G. The existence of degradation products capable of this conversion have been shown in samples of stored VX.[3]

In the event of a possible nerve agent exposure, a method would be needed to analyze for both G-agents and V-agents in biological matrices. Treatment eight was similar to a method previously used on archived serum samples to confirm human exposure to GB.[1] In the present case of VX spiked human serum this method produced the corresponding VX-G in measurable amounts indicating this method can be used to detect both the G and V series of nerve agents. Treatment eight also produced slightly higher amounts of VX-G in rat serum (3.95 ng/mL) as compared to treatment four which depended on endogenous fluoride levels (3.29 ng/mL).

Treating VX spiked human serum with KF and injecting the ethyl acetate through the silver fluoride pad yielded the highest amounts of VX-G (treatment seven, 12.7 ng/mL) which is probably a combination of free VX-G precursors and enzyme bound VX-G precursor. Rat serum extract did not produce the same effect upon injection through the pad.

Addition of buffer, which acidifies the sample to pH 4, appears to decrease the free VX-G precursor in the case of human serum as illustrated by the drop from 5.63 to 4.05 ng/mL. One possible explanation for this drop is the ionization of a VX-G precursor lessens its affinity for the C_{18} SPE

cartridge. For example, the amine group in VX would be ionized to a greater extent at pH 4 than at pH 7 creating a larger percentage of charged molecules that would be less likely to bind to the SPE media. The question of how VX or some closely related molecule survives the initial C_{18} SPE cartridge in the case of human serum and not rat serum is not clear. Additional investigation is required to elucidate this phenomenon.

Rat serum spiked at 176.6 ng/mL VX yielded 165.4 ng/mL of VX as G-analog in the filtrate of the SPE cartridge. This filtrate was injected on the silver fluoride pad and represents unbound or excess VX added to the serum. Aliquots of 0.25 mL of the filtered serum were then analyzed after being treated with reagents. These samples produced approximately 3.95 ng/mL of VX as G-analog. This represents the bound form of the nerve agent. The sum of both unbound and bound VX gives a reasonable mass balance of 169.4 ng/mL which accounts for 95.9% of the agent. It was noted that omission of the fluoride reagent in the preparation of the rat serum does not drastically effect the resulting VX-G production from the spiked samples as seen in Table 1 (3.29 ng/mL without fluoride versus 3.95 ng/mL with fluoride). This observation is in agreement with earlier reports that noted rat serum had endogenous levels of fluoride sufficient to regenerate Soman (GD) on addition of acetate buffer.[4,5]

CONCLUSIONS

A method for the verification and quantification of a nerve agent biomarker was developed for VX. VX exposure can be verified as G-analog in serum samples. This analysis is simple and quick using instrumentation available in field laboratories and air monitoring facilities and it is applicable to G-series and V-series nerve agents. The major disadvantage is that the original leaving group is not known but this is also true in the case of the agent metabolites.

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